bio-protocol Preprint

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Library construction and single-clone sequencing

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An abbreviated version of this protocol was published in Science Advances in Aug 2021

Rapid generation of maternal mutants via oocyte transgenic expression of CRISPR-Cas9 and sgRNAs in zebrafish

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Detailed protocol

Library construction and single-clone sequencing

Plasmid linearization
1, Prepare the enzyme digestion mixture:

PCS2-MT	1 μg
BamHI (10 U/μL, Thermo Scientific, ER0051)	1 μL
10X BamHI buffer	2 μL
H_2O	up to 20 μL

2, Incubate the mixture at 37°C for 2 hours.
3, Take 1 μL of the mixture to run agarose gel electrophoresis. The undigested plasmid was loaded as a negative control. Make sure that the plasmid was thoroughly digested.
4, Purify the linearized plasmid by DNA purification kit (Axygen, AP-PCR-250G).
5, Measure the concentration of purified plasmid using a nucleic acid quantification machine (Such as Allsheng, nano-200, or other equivalents).
6, Store the digested plasmid in -20°C.

CDS amplification
1, Design the primers for Gibson assembly manually or via Graphpad software. The CDS fragment amplified by this pair of primers should contain the initiation codon and exclude the stop codon. The ligated wild-type CDS sequence should be in frame of the 6X-myc sequence in the pCS2-MT plasmid.
2, Amplify the CDS from the cDNA using high fidelity DNA polymerase (Vazyme, P505-d1). The PCR mixture was formulated as follow:

2X Phanta Buffer	25 μL
dNTP mixture (10 mM each)	1 μL
CDS forward primer	2 μL
CDS reverse primer	2 μL
Phanta DNA polymerase	1 μL
cDNA	1 μL
ddH_2O	up to 50 μL

The PCR procedure was:

1	95°C	3 min
2	95°C	15 s
3	Tm	15 s
4	72°C	30 s / 1 kb
5	Jump to 2	34 times
6	72°C	5 min
7	25°C	10 min

7	25°C	10 min

3, Check the PCR products by agarose gel electrophoresis.
4, Purify the PCR products by DNA purification kit (Axygen, AP-PCR-250G).
5, Measure the concentration of purified PCR products using a nucleic acid quantification machine (Allsheng, nano-200).

Gibson assembly
1, Combine the CDS fragment with the linearized PCS2-MT using the Gibson assembly kit (Yeason, 10911ES20) following the instruction:
2, Make the mixture as follow:

2X Hieff clone enzyme premix	10
Linearized PCS2-MT	50 ng
CDS fragment	Molar ratio (plasmid : CDS)=1 : 3
ddH_2O	up to 20 μL

3, Incubate at 50°C for 20 min.
4, Put it on ice for 5 min.

Transform the DNA into the bacteria.
1, Take 100 μL DH5α competent cell from the -80°C refrigerator and thaw on ice.
2, Add 10 μL ligation mixture and mix them gently.
3, Incubate for 30 min on ice.
4, Heat-shock the competent cell at 42°C for 60 s.
5, Put it on ice immediately and incubate for 2 min.
6, Spread it on LB solid medium containing 100 μg/mL ampicillin.
7, Incubate at 37°C for 14 h.

Colony PCR
1, Pick up a certain number of colonies as you want. Mix each colony with 10 μL of ddH_2O in separate tubes, and that will serve as the DNA template in the following colony PCR.
2, Make colony PCR mixture:

2X Taq master mixture	5 μL
CDS forward primer	0.4 μL
CDS reverse primer	0.4 μL
Diluted bacteria	2 μL
ddH_2O	up to 10 μL

3, The PCR procedure:

1	94°C	5 min
2	94°C	30 s
3	Tm	30 s
4	72°C	30 s / 1 kb
5	To 2	22 times
6	72°C	7 min
7	25°C	10 min

4, Run colony PCR products on agarose gel electrophoresis,
5, Pick up colonies that have positive bands and expand them.

Bacteria expanded culture
1, Add the remaining bacteria to 1.8 mL LB liquid medium containing 100 μg/mL ampicillin in 24-well cell culture plates.
2, The bacteria medium was cultured at 37°C for 14 h. The rotational speed of the shaker was 200 r/min.

Sanger sequencing
The bacteria culture of each colony was subjected to Sanger sequencing. The sequencing company will extract plasmids separately and apply them to Sanger sequencing.

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Zhang, C. and 邵, 明. (2022). Library construction and single-clone sequencing. Bio-protocol Preprint. bio-protocol.org/prep1520.
2. Zhang, C., Lu, T., Zhang, Y., Li, J., Tarique, I., Wen, F., Chen, A., Wang, J., Zhang, Z., Zhang, Y., Shi, D. and Shao, M.(2021). Rapid generation of maternal mutants via oocyte transgenic expression of CRISPR-Cas9 and sgRNAs in zebrafish . Science Advances 7(32). DOI:

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